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13. ABSTRACT ***(Maximum 200 Words)***

The working hypothesis of the present project is that hyaluronan (HA) is responsible for maintaining pericellular spaces between nests of prostate tumor cells that are vital for the diffusion of nutrients required for tumor growth. To test this hypothesis, the cDNA for human HA synthase 3 (HAS3) was cloned and transfected into TSU cells which then over-express HA. These transfected cells were found to differ from their vector transfected counterparts with respect to the following: 1) they grew at a faster rate in high (but not low) density cultures; 2) conditioned media from these cells stimulated the proliferation and migration of endothelial cells; 3) when placed on the chorioallantoic membrane of chicken embryos, these cells formed large, dispersed xenografts while the control transfectants formed compact masses; and 4) when injected subcutaneously into nude mice, the xenografts formed by HAS3 transfectants were bigger than those formed by control transfectants. Histological examination of these xenografts indicated that the HAS3 transfectants had increased intercellular space rich in HA, and a greater number of blood vessels. These results support our working hypothesis that expression of HA promotes tumor cell growth.

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Charles Tinderhill 1/25/00

PI - Signature Date

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INTRODUCTION:

The working hypothesis of the present proposal is that hyaluronan (HA) associated with prostate tumor cells is responsible for maintaining a pericellular space between the nests of cells that is vital for the diffusion of nutrients. Without this pericellular space, tumor cells located only a short distance away form the blood supply will not receive an adequate supply of nutrients and will undergo necrosis. To test this working hypothesis, we have proposed the following specific aims:

1. We will examine the effects of increased HA production on tumor growth. For this we will transfect TSU cells (derived from human prostate cancer) with an expression vector for HA synthase, and the transfected clones as well as controls will be injected into nude mice. If our hypothesis is correct, then clones transfected with HA synthase will grow in the nude mice, while the control clones will not.
2. We will examine the effects of down regulating the synthesis of HA on the growth of prostate tumor cells. For this, we will examine the human prostate cell lines PC3 or TSU that produce large amounts of HA and readily grow in nude mice. These cell lines will be transfected with an antisense vector for HA synthase and then injected into nude mice along with control clones. If our hypothesis is correct then the transfected cells will grow at a slower rate and have less vascularization than the control cells. The transfected clones as well as control clones will be injected into nude mice. If our hypothesis is correct, then the hyaluronidase transfected cells should have a reduced growth rate as compared to control cells.
3. We will examine the effects of removing pericellular HA with hyaluronidase. The PC3 and TSU cell lines will be transfected with expression vectors for cell surface hyaluronidase and then injected into nude mice. Again, if our hypothesis is correct then the transfected cells should grow at a slower rate than the control cells.

BODY

A number of studies have suggested that the production of hyaluronan (HA) is associated with the metastatic behavior of tumor cells (1-11). Base on a variety of evidence, we believe that pericellular HA can enhance tumor cell growth by maintaining channels in the extracellular matrix, through which nutrients can diffuse. To test this possibility, we have proposed to manipulate the production of HA by tumor cells and examine the effect on their behavior.

During the initial funding period, we have concentrated our efforts on task 1, which involves transfecting tumor cells with expression vectors for HAS3, one of the enzymes that is responsible for the synthesis of HA and is located on the inner surface of the plasma membrane (12-19). We have accomplished most of the original goals and are presently preparing a manuscript describing the results of this study. In addition, we have begun studies involved in Tasks 2 and 3 and the progress will be briefly described.

TASK 1. The Effects of Increased HA Expression on Tumor Growth: To test our working hypothesis that HA promotes the growth of tumor cells, we have transfected a human prostate cancer cell line with an expression vector for HA synthase. Initially, we had proposed to the LNCaP cell line, however in preliminary studies we found that these cells do not readily grow in CAM of chicken embryos or in nude mice. For this reason, we have switched to the TSU cell line that readily grows in these two model systems. These cells were transfected with expression vectors containing candies for mammalian HA synthase 3 that we isolated from a library of human brain cDNA.

1.1. Isolation of cDNA for HAS-3: The full length cDNA for HAS3 was cloned from a cDNA library of human brain using a probe consisting of the partial sequence of the gene. The open reading frame contained 1,659 base pairs corresponding to 553 amino acids as shown in Fig. 1. The HAS3 protein has a deduced molecular weight about 63 kDa and an isoelectric point of 8.70. The first 27 amino acid correspond to the signal peptide and there are six potential transmembrane domains. Between the first (amino acid 43 to 65) and second transmembrane domains (amino acid 384 to 402), there is a stretch of 317 amino acids, which may be the major functional region for polysaccharide synthase. The remaining 170 amino acids in the C terminal region (from amino acid 403 to 553) contain 4 transmembrane domains that can form two loops spanned in and out of membrane. There is a potential site for N-glycosylation site on amino acid 462, a glycosaminoglycan attachment site on position 464, and several phosphorylation sites for tyrosine kinase, casein kinase, protein kinase C and cAMP-and cGMP-dependent protein kinase. Comparison with related enzymes indicates that human HAS3 has about 53% identity to HAS1 (both human and mouse form), 67% identity to HAS2 and 96% identity to mouse HAS3. HAS3 also contains seven HA-binding motifs of $B(X_7)B$ in which B is either R or K and X_7 contains no acidic residues and at least one basic amino acid (25). Similar domains are present in other HA-binding proteins such as RHAMM, CD44, hyaluronidase, link protein, aggrecan, human GHAP and TSG-6. These results are consistent with earlier studies of this and related genes (13-19).

1.2. Expression of HA in TSU cells by transfection of human HAS3. To examine the role of HAS3 in tumor progression, the cloned cDNA was inserted into a mammalian expression vector (pcDNA3) under the control of a CMV promoter and transfected into TSU (human prostate cancer) cells. To avoid complications associated with clonal variations, all of the clones that survived in 1 mg/ml of G418 were pooled and expanded for experimental analysis throughout this study.

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  1 ATG CCG GTG CAG CTG ACG ACA GCC CTG CGT GTG GTG GGC ACC AGC CTG TTT GCC CTG GCA 60
  1 M   P   V   Q   L   T   T   A   L   R   V   V   G   T   S   L   F   A   L   A  20

 61 GTG CTG GGT GGC ATC CTG GCA GCC TAT GTG ACG GGC TAC CAG TTC ATC CAC ACG GAA AAG 120
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121 CAC TAC CTG TCC TTC GGC CTG TAC GGC GCC ATC CTG GGC CTG CAC CTG CTC ATT CAG AGC 180
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 81 S   P   R   R   G   S   V   A   L   C   I   A   A   Y   Q   E   D   P   D   Y 100

301 TTG CGC AAG TGC CTG CGC TCG GCC CAG CGC ATC TCC TTC CCT GAC CTC AAG GTG GTC ATG 360
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541 AGC ACC TTC TCG TGC ATC ATG CAG AAG TGG GGA GGC AAG CGC GAG GTC ATG TAC ACG GCC 600
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261 V   R   Y   W   M   A   F   N   V   E   R   A   C   Q   S   Y   F   G   C   V 280

841 CAG TGT ATT AGT GGG CCC TTG GGC ATG TAC CGC AAC AGC CTC CTC CAG CAG TTC CTG GAG 900
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1021 ACA GAG ACC CCC ACT AAG TAC CTC CGG TGG CTC AAC CAG CAA ACC CGC TGG AGC AAG TCT 1080
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401  F   Y   R   G   R   I   W   N   I   L   L   F   L   L   T   V   Q   L   V   G 420

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1321 CTC TAC TCC CTC CTC TAT ATG TCC AGC CTT CTG CCG GCC AAG ATC TTT GCC ATT GCT ACC 1380
441  L   Y   S   L   L   Y   M   S   S   L   L   P   A   K   I   F   A   I   A   T 460

1381 ATC AAC AAA TCT GGC TGG GGC ACC TCT GGC CGA AAA ACC ATT GTG GTG AAC TTC ATT GGC 1440
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1501 TGC CAG GAC CTG TTC AGT GAG ACA GAG CTA GCC TTC CTT GTC TCT GGG GCT ATA CTG TAT 1560
501  C   Q   D   L   F   S   E   T   E   L   A   F   L   V   S   G   A   I   L   Y 520

1561 GGC TGC TAC TGG GTG GCC CTC CTC ATG CTA TAT CTG GCC ATC ATC GCC CGG CGA TGT GGG 1620
521  G   C   Y   W   V   A   L   L   M   L   Y   L   A   I   I   A   R   R   C   G 540

1621 AAG AAG CCG GAG CAG ACA AGC TTG GCT TTT GCT GAA GTG TGA 1662
541  K   K   P   E   Q   T   S   L   A   F   A   E   V   *  553
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Fig. 1. Structural characteristics of human HAS3. The complete nucleotide sequence and the deduced amino acid sequences of human HAS3 are shown.

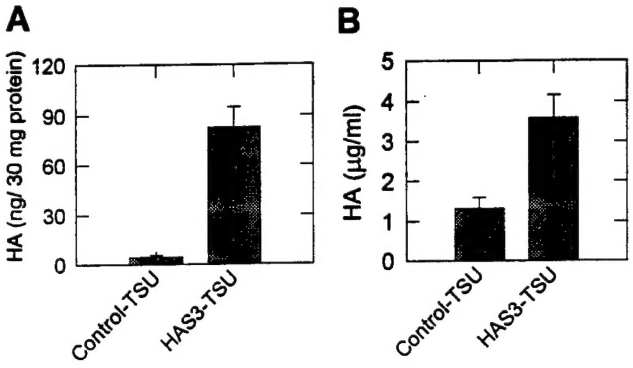

Fig. 2. Production of HA by vector control and HAS3 transfected TSU cells. Both control and HAS3 transfected cells were grown to confluence and then the cell layer and medium were analyzed for HA content using a modified enzyme linked assay. A. The amount of cell layer associated HA (normalized to the protein concentration) was higher in the HAS3TSU cells than in the control TSU cells. B. The amount of HA secreted into the medium by the HAS3-TSU cells was greater than that of the control-TSU cells. Note that most of the HA was secreted into the medium.

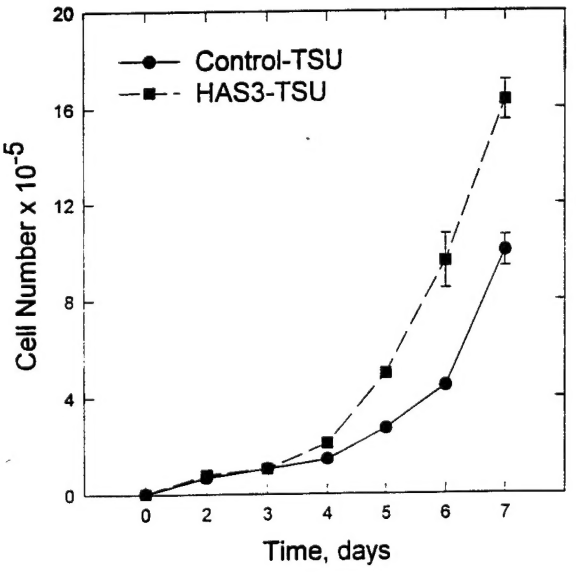

Fig. 3. The proliferation of control and HAS3 transfected TSU cells. The cell lines were cultured 24 well dishes in complete medium, that was changed ever other day. At the indicated time, a set of wells were harvested with a solution of EDTA in PBS and the cell numbers were determined with a Coulter counter. At low densities, no differences in the growth rates were apparent. However, at higher densities, the HAS3 transfected cells grew at a faster rate. Three replicates of this experiment gave similar results.

The production of HA by these cells was examined by a modified enzyme linked assay. As shown in Fig. 2, HAS3-TSU cells produced significantly greater amounts of medium and cell-associated HA than the control-TSU cells. These finding were confirmed by dot blotting of the conditioned medium onto nitrocellulose followed by peroxidase-linked HA staining (data not shown). It is important to note that the amount of secreted HA was significantly higher than that associated with the cell layer.

Cultures of the vector and HAS3 transfected TSU cells were then compared with respect to their HA staining pattern. At low densities, there was little obvious difference between the cell types, both of which expressed similar levels of HA that was present in the cytoplasm and on the cell surface (data not shown). Presumably this was due to the endogenous HA synthase present in the TSU cells that is active in the growing cells and masks the effect of the transfected HAS3. However, at high densities significant differences became apparent (Fig. 3). The control-TSU cells displayed a cobble-stone appearance indicative of contact inhibition of growth, and the production of HA was significantly down regulated (Fig. 3 A). In contrast, the HAS3-TSU cells appeared to have lost contact-inhibition of growth, forming numerous multi-layered clusters of cells, with which most of the HA staining was associated (Fig. 3 B). We believe that in these higher density cultures, the endogenous HA synthase is down-regulated due to contact inhibition of growth (26-29) and under these conditions the constitutive expression of the transfected HAS3 gene (controlled by the CMV promoter) becomes more prominent.

Taken together, the results of this section indicate that the transfection of HAS3 into TSU cells stimulates their expression of HA.

1.3. HAS3 Promotes Cell Growth at High Densities. We then compared the growth rates of the control and HAS3 transfected cells. For this, the cells were transferred to multi-well dishes, and at various times the cells were harvested and enumerated with a Coulter counter. Figure 4 shows that during the initial stages of the assay while the cells were still at low density, there was no obvious difference in the proliferation rates of the control and HAS3 transfected cells. However, beginning on day 4, when the cell densities were higher, the HAS3-TSU cells proliferated at a faster rate that the control cells. Similar results were obtained in three separate replications of this experiment. Thus, at high densities, the HAS3 transfected cells grow at a faster rate, presumably because they has lost part of their contact inhibition of growth and have the ability to form multi-layered clusters.

As indicated earlier, transfection of cells with HAS3 increase the levels of HA in both the medium and the cell-layer fractions, either of which could be responsible for the enhanced growth rate. To distinguish between these two fractions, we added varying concentrations of highly purified HA (2 to 200 μg/ml) to the culture medium of the transfected cells. At the end of 7 days, no obvious difference was apparent in the cell number of either cell type (data not shown). Thus, the presence of free HA by itself does not stimulate the cell growth under these conditions and suggests that increased levels of HA secreted into the medium does not act in an autocrine or paracrine fashion to stimulate the growth of these cells. A more likely explanation is that the cell-associated fraction of HA (cell surface or cytoplasmic) plays a more important role in regulating the growth of these cells at high density.

1.4. Effect of Conditioned Media from HAS3-TSU on Endothelial cells: Since several studies have reported that under certain conditions, HA can modulate angiogenesis (30, 31) we examined the effects of conditioned medium from HAS3-TSU cells on the behavior of cultured endothelial (ABAE) cells. As shown in Fig. 5 A, conditioned medium from HAS3-TSU cells stimulated the proliferation of the ABAE cells by 66% as compared to that from control cells as judged by ^{3}H-TdR incorporation. Furthermore, the conditioned medium from the HAS3-TSU also stimulated the migration of ABAE cells through

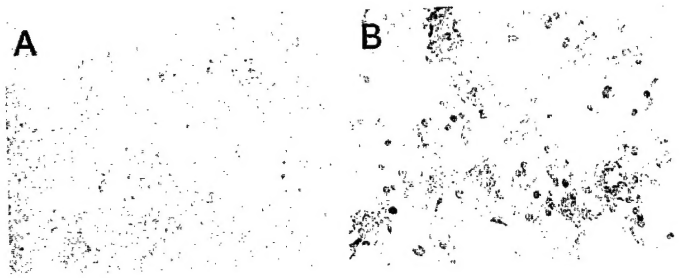

Fig. 4. Histochemical staining of control and HAS3-transfected TSU cells for HA using a biotinylated HA binding probe from cartilage. High density cultures were fixed, and incubated with b-HABP, followed by peroxidase labeled streptavidin and finally a substrate that gives rise to a red reaction product. A. A high density culture of control-TSU cells reveals a cobble stone appearance characteristic of cells exhibiting contact inhibition of growth . These cells have very little HA staining associated with them. B. A high density culture of HAS3-TSU cells reveals the presence of multilayered clusters of cells indicative of diminished contact inhibition of growth. Most of the HA staining was associated with these clusters.

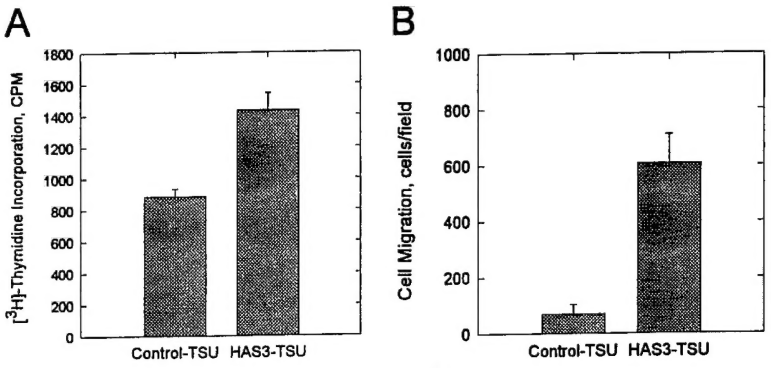

Fig. 5. Effects of conditioned medium on the proliferation and migration of endothelial cells. A. Cultures of ABAE cells were grown in the presence of conditioned from either control- or HAS3-TSU cells. After 36 hr [^{3}H]-thymidine was added to the medium for 8 hours, the cells were harvested and the amount of incorporated radioactivity was determined. The conditioned medium from the HAS3-TSU cells had a greater stimulatory effect on the proliferation of the ABAE cells than that of the control-TSU cells. B. To examine cell migration aliquots of ABAE cells were added to a 48 well Boyden chamber, allowed to attach to a Nucleopore membrane and then conditioned medium was added to the wells on the opposite side of the membrane. After 2 hours, the cells that had migrated across the filter were stained, and enumerated (10 random fields were counted). The conditioned medium from the HAS3-TSU cells stimulated the migration of the endothelial cells to a greater extent than that from the control-TSU cells.

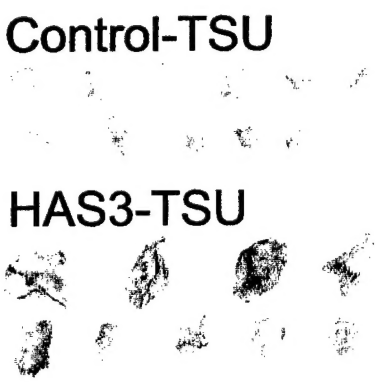

Fig. 6. Growth of control and HAS3-TSU cells on the chicken CAM. Control and HAS3-transfected cells were placed on the CAMs of 10 day-old chicken embryos (1 x 10^6 cells/egg) and incubated at 37 °C. Five days later, the xenografts were removed and photographed: top panel, control-TSU cells; lower panel, HAS3-TSU cells.

Nucleopore filters as compared to medium from that from control cells by more than 600% (Fig. 5 B). These results further suggest that the excess of HA produced by HAS3 in conditioned media from TSU cells could exert the stimulatory function on endothelium and is consistent with the early studies of West and coworkers (30, 31).

1.5. Growth of HAS3-TSU Cells on the CAM: Next, we compared the growth characteristics of the control and HAS3 transfected cells on the CAM of chicken eggs. In this experiment, equal numbers of the transfected TSU cells were placed on the CAMs of 10 day-old chicken embryos and allowed to grow for 5 days. As shown in Fig. 6, at the end of this period, the xenografts showed a striking divergence in their morphology. The control TSU cells formed rounded, nodular xenografts, with necrotic tissue in the center. In contrast, the HAS3-TSU cells gave rise to xenografts with a dispersed or spread-out morphology and without any obvious necrosis (Fig. 6). The HAS3-TSU xenografts were also 156 % larger than those of the control-TSU cells (Table 1). These results suggest that over-expression of HA enhances the tumor growth on CAM system.

1.6. HAS3 Promotes TSU Growth in Nude Mice. The results from chicken CAM were further tested with nude mice. In this system, transfected cells were injected subcutaneously into nude mice (male, 5 week-old). Again, the xenografts formed by the HAS3-TSU cells grew at a faster rate and appeared to be more vascularized than the control cells. After 3 weeks of growth, the HAS-3 xenografts were 3 times larger than those formed by the control cells (Fig. 7, Table 1). This suggests that HAS3 promotes the growth of TSU tumor cells in mice and is consistent with the results from the chicken CAM system.

However, when transfected cells were injected via tail vein into nude mice (5 mice/group), no lung metastasis was detected with either cell line. Thus, the over-expression of HA by itself is not sufficient to induce the metastatic behavior in these cells.

1.7. HAS3 Increases Intercellular Space and Angiogenesis in HAS3 Xenografts. Histological examination of the xenografts from nude mice revealed differences in their morphology (Fig. 8). While the xenografts varied from region to region, in general, the cells in the control-TSU xenografts were relatively homogeneous, compact and most of the HA appeared to be present in the cytoplasm of the cells (Fig. 8 A). In contrast, the HAS3-TSU cells formed small clusters or nests of cells that were surrounded by a matrix rich in HA (Fig. 8 B). Such structures were not observed in the control-TSU xenografts.

The extent of angiogenesis in these xenografts was examined histochemically staining for endothelial cells (anti-CD31). The HAS3-TSU xenografts demonstrated stronger staining for endothelial cells as compared to the control-TSU xenografts. The number of vessels in random 10 high-power fields in the samples of HAS3-TSU xenografts was 138% higher than that from control-TSU cells (169 in Control-TSU xenografts vs. 403 in the HAS3-TSU xenografts). This suggests that increased levels of HA can stimulate angiogenesis in these cell and this may, in part, account for the faster growth rate.

1.8. Interpretation of Results: In this study, we have characterized human HAS3 with regard to both its structure and its function in tumor progression. Based upon its deduced amino acid sequence, HAS3 shares significant homology with HAS1 and HAS2, and contains a signal peptide as well as six transmembrane regions strongly suggesting that it associated with the plasma membrane. Its enzymatic activity was demonstrated by the fact that transfection of TSU cells with expression vectors resulted in increased production of HA as determined by histochemical staining, dot blot analysis and quantitative ELISA. These findings are consistent with earlier studies of HAS proteins (12-19)

Table 1. The growth of transfected TSU cells in the chicken CAM and in the nude mouse. The TSU cells were grown on the CAM for 5 days were carefully removed and weighed. In nude mouse model system, the TSU cells were grown for 22 days and the volumes were determined.

	Xenograft		
	Control-TSU	HAS3-TSU	% Increase
Chicken CAM (5 days)	59.1±5.6 mg	151.3±23.7 mg	156
Nude Mice (22 days)	0.063±0.016 cm^3	0.311±0.049 cm^3	394

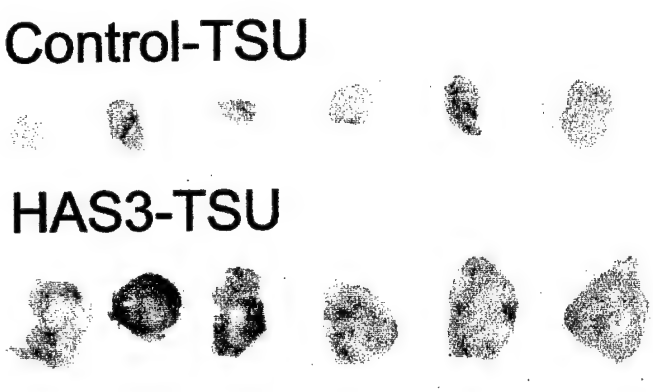

Fig. 7. Xenografts of control and HAS3 transfected TSU cells in nude mice. Nude mice were injected with 2 x 106 control- and HAS3-TSU cells. Twenty-two days later, the xenografts were harvested and photographed: top panel, control-TSU cell; lower panel, HAS3-TSU cells.

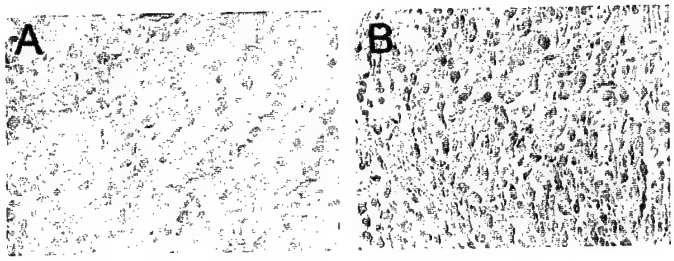

Fig. 8. The distribution of HA in xenografts formed by transfected TSU cells in nude mice. The xenografts were fixed in formalin, processed for histology and the resulting paraffin sections were stained for HA (red) using b-HABP and counter-stained with hematoxylin (blue). A. A representative section of a control-TSU xenograft shows that cells are compact and most of the HA staining is associated with the cytoplasm of the cells. B. A representative section of a HAS3-TSU xenograft reveals that cells are present in small clusters, surrounded by a stroma rich in HA. While the microscopic morphology of the xenografts varied from region to region, the HA-rich stroma was prevalent in the HAS3-TSU cells and absent from the control-TSU cells.

This study also suggests that stimulation of HA synthesis in TSU cells by transfecting them with HAS3 expression vectors enhanced their growth in both chicken embryos and in nude mice. Furthermore, this enhanced tumor growth appears to be due to two distinct mechanisms. The first mechanism involves a direct effect on the tumor cells themselves as suggested by the fact that in tissue culture, HAS3 transfected cells grew at a faster rate at high density. The second mechanism promoting tumor growth rate results from an increase in vascularization as reflected by the greater density of blood vessels present in xenografts of transfected cells in nude mice. Together, these two factors contribute to the increased tumor growth rate.

While HAS3 stimulates the production of both secreted and cell-associated HA, it is this latter fraction that appears to be most important in modulating cell growth in culture. This was suggested by the observation that addition of free HA to the cell medium did not result in increased proliferation that was seen in the HAS3 transfected cells. Thus, the most likely explanation is that it is the cell-associated pool of HA that is responsible for the biological effects on proliferation. This cell-associated fraction of HA consists of that present in the cytoplasmic as well as on the cell surface, either or both of which could be responsible for the increase in proliferation.

The HA on the surface of the cells can form a pericellular coat which can be directly visualized by its ability to exclude small particles such as erythrocytes (32). The size of this coat is due in part to small, microvilli-like projections that extend out from the surface of the cells to which the HA is attached (33). This pericellular coat could stimulate the growth of cells by several different mechanisms. One possible mechanism is that it disrupts intercellular junctions and thereby allows the cells to detach from the substrate so that they can divide and occupy new space (34-36). This would allow cells to overcome contact inhibition of growth which is characteristic of TSU cells and allow them to from multi-layers at high density cultures as we have observed. Another possible mechanism by which pericellular HA could stimulate proliferation is by maintaining spaces between cells that increases the flow of nutrients. Indeed, the larger extracellular space apparent in xenografts of HAS3 transfected cells in nude mice could serve as conduits through which nutrients could diffuse to support cells located some distance from the blood supply and thus facilitate their growth. Presumably, the

HA also appears to promote vascularization which is clearly important in regulating tumor growth (37-40). This was indicated by our observation that in xenografts of HAS3-TSU cells formed in nude mice, there was an increase in the density of blood vessels as compared to the control cells as determined by histochemical staining for endothelial cells (CD31). Part of this increased vascularization may be due to the pericellular spaces generated by the HA provides space which facilitates the migration and invasion of endothelial cells. In addition, the HA itself can stimulate the migration of endothelial cells. This was also indirectly suggested by experiments showing that conditioned media from the HAS3 transfected cells stimulated the growth and migration of cultured endothelial cells. This is consistent with earlier studies of West and his associates have shown that oligosaccharide fragments of HA stimulate the formation of new blood vessels in the chorioallantoic membrane of chicken embryos (30, 31).

While the results of this suggest that over-expression of HAS3 in TSU prostate cancer cells promotes their tumorigenicity, there are some aspects of the present study that appear to contradict the results of other studies. For example, while we found that TSU transfectants grew faster in culture, Kosaki and coworkers found no such increase in the growth of HT1080 cells transfected with HAS2 under anchorage dependent conditions, although these cells did form bigger colonies in suspension culture (20). In addition, while we found increased stroma and angiogenesis in xenografts of HAS3-TSU cells in nude mice, Kosaki *et al.* found no such increase in HAS2 transfected HT1080 cells (20). We believe that these difference may due

in part to the different target cells that were employed in these studies. The TSU cells employed in this study are of epithelial origin, while HT1080 cells are derived from a fibrosarcoma of connective tissue cells. This could account for the differences see with regard to growth behavior and the production of HA in the connective tissue, and their ability to stimulate angiogenesis. Alternatively, the differences could be attributed to the characteristics of the HA synthases that were used in the transfection, since they differ with respect to both their synthetic activity as well as the size of the HA that they produce (16-18).

Another apparent discrepancy was our observation that transfection of TSU cells with HAS3 did not appear to stimulate their ability to form lung metastases in nude mice. In contrast, we had previously reported that the levels of HA on cell surface of B16 cells was directly correlated with their metastatic behavior (3). Similarly, Itano *et al.* found that transfection of FM3A with HAS1 enhanced their metastatic properties (21). Again, we believe that these divergent results are due to the different cell types that are used as targets for transfection. In the case of B16 and FN3A, these cell lines originally possess the ability to undergo metastasis, and stimulation of HA synthesis in these cells enhance this innate property. In contrast, TSU cells appear to lack this ability (at least in nude mice) and increased HA synthesis, by itself, is not sufficient to promote metastatic properties. Clearly, the process of metastasis is a complex phenomenon involving the collaboration of many molecules. While the production of HA is one of the factors, it is not sufficient for the tumor metastasis.

In conclusion, the results of this study indicates that HA expression plays a role in tumor progression and are consistent with earlier studies demonstrating a correlation between HA levels and tumorigenicity. Furthermore, the HA may be acting through several different mechanisms, including: 1, a direct effect on the growth of the tumor cells themselves; 2, the formation of extracellular conduits through which nutrients can flow; and 3, the stimulation of blood vessel growth. However, these effects depend upon the particular cell type and the specific environment. Given the complexity of the effects of HA, it may be difficult to predict exactly how HA will influence the behavior of tumor cells. In any case, HA be more of a facilitator or promoter of tumor growth than an instigator.
If our working hypothesis is correct, then transfection of cells with HA synthase should promote their ability to grow in nude mice. Histological examination of any resulting growth will be used to confirm the expression of HA.

TASK 2. The Effects of Decreased HA Expression on Tumor Growth: If HA plays an important role in tumor growth, then blocking of the production of HA should inhibit the malignant progression. To test his possibility, we have transfected prostate and breast cancer cell lines cancer with the vectors that drive the expression of antisense RNA for these genes. The antisense RNA binds specifically to the mRNAs for HA synthase, target these molecules for degradation and inhibit the normal translation of HA synthase. The resultant were then examined for the phenotype changes in terms of growth rate in nude mice and the degree of vascularization. The target cell lines for these studies will be TSU and MDA231 cells. Since this study is ongoing, our progress will only be briefly summarized here.

2.1. Transfection of Tumor Cells with Vectors for the Antisense of HA Synthase: Antisense expression vectors for HAS3 were prepared and transfected into both TSU cells (human prostate cancer) and MDA-231 cells (human breast cancer). The transfected cells were selected and found to express lower levels of HA (since the sequence of HAS3 is very similar to that of HAS1 and 2, antisense toHAS3 is able to down-regulate all three isoforms of HA synthase).

2.2. Examine the Effect of Antisense on Tumor Growth and Angiogenesis: When injected subcutaneously into nude mice, both the TSU and MDA231 cells transfected with antisense to HAS3 formed smaller tumors than their control counterparts (i.e. transfected with sense sequence to HAS3). These results are again consistent with our working hypothesis that HA can promote tumor cell growth. In the immediate future, the resulting xenografts will be further examined histochemically to determine if there

TASK 3. The Effect of Hyaluronidase on the Growth of Tumor Cells: In this task, we will use a different approach the reduce the levels of pericellular HA associated with tumor cells. For this, prostate cancer cells have been transfected with an expression vector for hyaluronidase (sperm-type and membrane-bound, PH-20) which will degrade the HA so that it will not accumulate in the pericellular matrix.

3.1. *Preparation of the pCHisC-PH-20 expression vector:* The pCHisC-PH-20 was amplified in bacteria and the purified vector will be transfected into TSU cells using the calcium precipitation method. The transfected clones were selected and expanded for further characterization. These cells were found to have reduced levels of HA as compared to control cells.

3.3. Assessment of Tumor Growth Rates and Angiogenesis: Samples of the transfected cell lines were injected sc. into nude mice and the resulting xenografts were found to be smaller than those from control transfected cells. Again, this is consistent with our working hypothesis. In the immediate future, these xenografts will be examined for the extent of angiogenesis.

ACRONYMS AND SYMBOL DEFINITIONS

b-HABP	Biotinylated proteoglycan - used as a specific staining probe for hyaluronan.
CAM	Chorioallantoic membrane
CD44	Cluster of determination (differentiation) - same as the hyaluronan receptor or binding site.
CMF-PBS	Calcium and magnesium free phosphate buffered saline.
DMEM	Dulbecco's modifed Eagle's medium
HA	Hyaluronan.
HAase	Hyaluronidase (either testicular or *Streptomyces*)
HAS3	Hyaluronan synthase, isoform 3
mAb	Monoclonal antibody.
HABP	A complex of a trypsin fragment of cartilage proteoglycan and link protein that binds to HA with high affinity and specificity.

KEY RESEARCH ACCOMPLISHMENTS:

- The cDNA for human HAS3 was cloned and characterized. The open reading frame consisted of 1,659 base pairs coding for 553 amino acids with a deduced molecular weight about 63 kDa and isoelectric pH of 8.70. The sequence of human HAS3 displayed a 53% identity to HAS1 and 67% identity to HAS2. It also contained a signal peptide and 6 potential transmembrane domains, suggesting that it is associated with the plasma membrane.
- To evaluate the physiological role of human HAS3, expression vectors for this protein were transfected into TSU cells (a prostate cancer cell line). The over-expression of HA in the transfected cells was confirmed by histochemical staining, dot blot analysis and ELISA.
- The HAS3-TSU cells were found to differ from their control transfected counterparts with respect to the following: 1) they grew at a faster rate in high (but not low) density cultures; 2) conditioned media from these cells stimulated the proliferation and migration of endothelial cells; 3) when placed on the chorioallantoic membrane of chicken embryos, these cells formed large, dispersed xenografts while the control transfectants formed compact masses; and 4) when injected subcutaneously into nude mice, the xenografts formed by HAS3 transfectants were bigger than those formed by control transfectants.
- Histological examination of these xenografts indicated that the HAS3 transfectants had increased intercellular space rich in HA, and a greater number of blood vessels.
- The HAS3 transfected TSU cells did not show an increase in their metastatic properties as judged by their ability to form lung metastases following i.v. injection.

REPORTABLE OUTCOMES:

The following manuscript will be submitted to *Cancer Research* in the immediate future:

Ningfei L., Gao, F., Han, H., Underhill, C. B. and Zhang, L. Hyaluronan Synthase 3 Expression Promotes the Growth of TSU Prostate Cancer Cells. To be submitted to *Cancer Research*.

CONCLUSION AND SIGNIFICANCE:

- These results suggest that the HAS3-induced over-expression of HA promotes tumorigenicity by several different mechanisms, including: 1) a direct effect on the growth of the tumor cells, 2) the formation of extracellular conduits through which nutrients can flow, and 3) the stimulation of blood vessel growth. This is consistent with our original working hypothesis that an extracellular matrix of HA enhances tumor cell growth.

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